PATENT
Docket No: 2160US

METHODS FOR THE IDENTIFICATION OF INHIBITORS OF PORPHOBILINOGEN DEAMINASE AS ANTIBIOTICS

RELATED APPLICATIONS

The present application claims the benefit of U.S. Provisional Application Serial No. 60/455,264, filed on March 17, 2003, herein incorporated in its entirety.

The present application is related to U.S. Application Serial No. 10/007,022, filed December 6, 2001, titled "Methods for the Indentification of Inhibitors of 5-Aminolevulinate synthase as Antibiotics," now issued U.S. Patent No. 6,689,578.

FIELD OF THE INVENTION

The invention relates generally to methods for the identification of antibiotics, preferably antifungals that affect the biosynthesis of heme.

BACKGROUND OF THE INVENTION

Filamentous fungi are causal agents responsible for many serious pathogenic infections of plants and animals. Since fungi are eukaryotes, and thus more similar to their host organisms than, for example bacteria, the treatment of infections by fungi poses special risks and challenges not encountered with other types of infections. One such fungus is *Magnaporthe grisea*, the fungus that causes rice blast disease, a significant threat to food supplies worldwide. Other examples of plant pathogens of economic

importance include the pathogens in the genera *Agaricus, Alternaria, Anisogramma, Anthracoidea, Antrodia, Apiognomonia, Apiosporina, Armillaria, Ascochyta, Aspergillus, Bipolaris, Bjerkandera, Botryosphaeria, Botrytis, Ceratobasidium, Ceratocystis, Cercospora, Cercosporidium, Cerotelium, Cerrena, Chondrostereum, Chryphonectria, Chrysomyxa, Cladosporium, Claviceps, Cochliobolus, Coleosporium, Colletotrichium, Colletotrichum, Corticium, Corynespora, Cronartium, Cryphonectria, Cryptosphaeria, Cyathus, Cymadothea, Cytospora, Daedaleopsis, Diaporthe, Didymella, Diplocarpon, Diplodia, Discohainesia, Discula, Dothistroma, Drechslera, Echinodontium, Elsinoe, Endocronartium, Endothia, Entyloma, Epichloe, Erysiphe, Exobasidium, Exserohilum, Fomes, Fomitopsis, Fusarium, Gaeumannomyces, Ganoderma, Gibberella, Gloeocercospora, Gloeophyllum, Gloeoporus, Glomerella, Gnomoniella, Guignardia, Gymnosporangium, Helminthosporium, Herpotrichia, Heterobasidion, Hirschioporus, Hypodermella, Inonotus, Irpex, Kabatiella, Kabatina, Laetiporus, Laetisaria, Lasiodiplodia, Laxitextum, Leptographium, Leptosphaeria, Leptosphaerulina, Leucytospora, Linospora, Lophodermella, Lophodermium, Macrophomina, Magnaporthe, Marssonina, Melampsora, Melampsorella, Meria, Microdochium, Microsphaera, Monilinia, Monochaetia, Morchella, Mycosphaerella, Myrothecium, Nectria, Nigrospora, Ophiosphaerella, Ophiostoma, Penicillium, Perenniporia, Peridermium, Pestalotia, Phaeocryptopus, Phaeolus, Phakopsora, Phellinus, Phialophora, Phoma, Phomopsis, Phragmidium, Phyllachora, Phyllactinia, Phyllosticta, Phymatotrichopsis, Pleospora, Podosphaera, Pseudopeziza, Pseudoseptoria, Puccinia, Pucciniastrum, Pyricularia, Rhabdocline, Rhizoctonia, Rhizopus, Rhizosphaera, Rhynchosporium, Rhytisma, Schizophyllum, Schizopora, Scirrhia, Sclerotinia, Sclerotium, Scytinostroma, Septoria, Setosphaera, Sirococcus, Spaerotheca, Sphaeropsis, Sphaerotheca, Sporisorium, Stagonospora, , Stemphylium, Stenocarpella, Stereum, Taphrina, Thielaviopsis, Tilletia, Trametes, Tranzschelia, Trichoderma, Tubakia, Typhula, Uncinula, Urocystis, Uromyces, Ustilago, Valsa, Venturia, Verticillium, Xylaria*, and others. Related organisms are classified in the oomycetes classification and include the genera *Albugo, Aphanomyces, Bremia, Peronospora, Phytophthora, Plasmodiophora, Plasmopara, Pseudoperonospora, Pythium, Sclerophthora*, and others. Oomycetes are also significant plant pathogens and are sometimes classified along with

the true fungi. Human diseases that are caused by filamentous fungi include life-threatening lung and disseminated diseases, often a result of infections by *Aspergillus fumigatus*. Other fungal diseases in animals are caused by fungi in the genera *Fusarium*, *Blastomyces*, *Microsporum*, *Trichophyton*, *Epidermophyton*, *Candida*, *Histoplamsa*, *Pneumocystis*, *Cryptococcus*, other *Aspergilli*, and others. The control of fungal diseases in plants and animals is usually mediated by chemicals that inhibit the growth, proliferation, and/or pathogenicity of the fungal organisms. To date, there are less than twenty known modes-of-action for plant protection fungicides and human antifungal compounds.

A pathogenic organism has been defined as an organism that causes, or is capable of causing disease. Pathogenic organisms propagate on or in tissues and may obtain nutrients and other essential materials from their hosts. A substantial amount of work concerning filamentous fungal pathogens has been performed with the human pathogen, *Aspergillus fumigatus*. Shibuya *et al.*, 27 *Microb. Pathog.* 123 (1999) (PubMed Identifier (PMID): 10455003) have shown that the deletion of either of two suspected pathogenicity related genes encoding an alkaline protease or a hydrophobin (rodlet), respectively, did not reduce mortality of mice infected with these mutant strains. Smith *et al.*, 62 *Infect. Immun.* 5247 (1994) (PMID: 7960101) showed similar results with alkaline protease and the ribotoxin restrictocin; *Aspergillus fumigatus* strains mutated for either of these genes were fully pathogenic to mice. Reichard *et al.*, 35 *J. Med. Vet. Mycol.* 189 (1997) (PMID: 9229335)) showed that deletion of the suspected pathogenicity gene encoding aspergillopepsin (PEP) in *Aspergillus fumigatus* had no effect on mortality in a guinea pig model system, whereas Aufauvre-Brown *et al.*, 21 *Fungal. Genet. Biol.* 141 (1997) (PMID: 9073488) showed no effects of a chitin synthase mutation on pathogenicity.

However, not all experiments produced negative results. Ergosterol is an important membrane component found in fungal organisms. Pathogenic fungi lacking key enzymes in the ergosterol biochemical pathway might be expected to be non-pathogenic since neither the plant nor animal hosts contain this particular sterol. Many antifungal compounds that affect the ergosterol biochemical pathway have been previously described. (United States Patent Nos. 4,920,109; 4,920,111; 4,920,112; 4,920,113; and 4,921,844; Hewitt, H. G. Fungicides in Crop Protection Cambridge,

University Press(1998)). D'Enfert *et al.*, 64 *Infect. Immun.* 4401 (1996) (PMID: 8926121)) showed that an *Aspergillus fumigatus* strain mutated in an orotidine 5'-phosphate decarboxylase gene was entirely non-pathogenic in mice, and Brown *et al.* (Brown *et al.*, 36 *Mol. Microbiol.* 1371 (2000) (PMID: 10931287)) observed a non-pathogenic result when genes involved in the synthesis of para-aminobenzoic acid were mutated. Some specific target genes have been described as having utility for the screening of inhibitors of plant pathogenic fungi. U.S. Patent No. 6,074,830 to Bacot *et al.*, describe the use of 3,4-dihydroxy-2-butanone 4-phosphate synthase, and U.S. Patent No. 5,976,848 to Davis *et al.* describes the use of dihydroorotate dehydrogenase for potential screening purposes.

There are also a number of papers that report less clear results, showing neither full pathogenicity nor non-pathogenicity of mutants. For example, Hensel *et al.* (Hensel, M. *et al.*, 258 *Mol. Gen. Genet.* 553 (1998) (PMID: 9669338)) showed only moderate effects of the deletion of the *areA* transcriptional activator on the pathogenicity of *Aspergillus fumigatus*. Therefore, it is not currently possible to determine which specific growth materials may be readily obtained by a pathogen from its host, and which materials may not.

The present invention discloses porphobilinogen deaminase (PBG) as a target for the identification of antifungal, biocide, and biostatic materials.

SUMMARY OF THE INVENTION

The present inventors have discovered that *in vivo* disruption of the gene encoding porphobilinogen deaminase in *Magnaporthe grisea* abolishes the pathogenicity of the fungus. Thus, the present inventors have discovered that porphobilinogen deaminase is useful as a target for the identification of antibiotics, preferably fungicides. Accordingly, the present invention provides methods for the identification of compounds that inhibit porphobilinogen deaminase expression or activity. The methods of the invention are useful for the identification of antibiotics, preferably fungicides.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Diagram of the reversible reaction catalyzed by porphobilinogen deaminase (PBG). The enzyme catalyzes the reversible interconversion of porphobilinogen and H_2O to hydroxymethylbilane and NH_3. This reaction is part of the heme biosynthesis pathway.

Figure 2. Digital image showing the effect of HEM3 gene disruption on *Magnaporthe grisea* pathogenicity using whole plant infection assays. Rice variety CO39 was inoculated with wild-type strain Guy11, transposon insertion strains, K1-6 and K1-23. Leaf segments were imaged at five days post-inoculation.

DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise indicated, the following terms are intended to have the following meanings in interpreting the present invention.

The term "antibiotic" refers to any substance or compound that when contacted with a living cell, organism, virus, or other entity capable of replication, results in a reduction of growth, viability, or pathogenicity of that entity.

The term "antipathogenic", as used herein, refers to a mutant form of a gene, which inactivates a pathogenic activity of an organism on its host organism or substantially reduces the level of pathogenic activity, wherein "substantially" means a reduction at least as great as the standard deviation for a measurement, preferably a reduction by 50%, more preferably a reduction of at least one magnitude, i.e. to 10%. The pathogenic activity affected may be an aspect of pathogenic activity governed by the normal form of the gene, or the pathway the normal form of the gene functions on, or the organism's pathogenic activity in general. "Antipathogenic" may also refer to a cell, cells, tissue, or organism that contains the mutant form of a gene; a phenotype associated with the mutant form of a gene, and/or associated with a cell, cells, tissue, or organism that contain the mutant form of a gene.

The term "binding" refers to a non-covalent or a covalent interaction, preferably non-covalent, that holds two molecules together. For example, two such molecules could be an enzyme and an inhibitor of that enzyme. Non-covalent interactions include hydrogen bonding, ionic interactions among charged groups, van der Waals interactions

and hydrophobic interactions among nonpolar groups. One or more of these interactions can mediate the binding of two molecules to each other.

The term "biochemical pathway" or "pathway" refers to a connected series of biochemical reactions normally occurring in a cell, or more broadly a cellular event such as cellular division or DNA replication. Typically, the steps in such a biochemical pathway act in a coordinated fashion to produce a specific product or products or to produce some other particular biochemical action. Such a biochemical pathway requires the expression product of a gene if the absence of that expression product either directly or indirectly prevents the completion of one or more steps in that pathway, thereby preventing or significantly reducing the production of one or more normal products or effects of that pathway. Thus, an agent specifically inhibits such a biochemical pathway requiring the expression product of a particular gene if the presence of the agent stops or substantially reduces the completion of the series of steps in that pathway. Such an agent, may, but does not necessarily, act directly on the expression product of that particular gene.

As used herein, the term "conditional lethal" refers to a mutation permitting growth and/or survival only under special growth or environmental conditions.

As used herein, the term "cosmid" refers to a hybrid vector, used in gene cloning, that includes a cos site (from the lambda bacteriophage). In some cases, the cosmids of the invention comprise drug resistance marker genes and other plasmid genes. Cosmids are especially suitable for cloning large genes or multigene fragments.

"Fungi" (singular: fungus) refers to whole fungi, fungal organs and tissues (*e.g.*, asci, hyphae, pseudohyphae, rhizoid, sclerotia, sterigmata, spores, sporodochia, sporangia, synnemata, conidia, ascostroma, cleistothecia, mycelia, perithecia, basidia and the like), spores, fungal cells and the progeny thereof. Fungi are a group of organisms (about 50,000 known species), including, but not limited to, mushrooms, mildews, moulds, yeasts, *etc.*, comprising the kingdom Fungi. They can either exist as single cells or make up a multicellular body called a mycelium, which consists of filaments known as hyphae. Most fungal cells are multinucleate and have cell walls, composed chiefly of chitin. Fungi exist primarily in damp situations on land and, because of the absence of chlorophyll and thus the inability to manufacture their own food by photosynthesis, are

either parasites on other organisms or saprotrophs feeding on dead organic matter. The principal criteria used in classification are the nature of the spores produced and the presence or absence of cross walls within the hyphae. Fungi are distributed worldwide in terrestrial, freshwater, and marine habitats. Some live in the soil. Many pathogenic fungi cause disease in animals and man or in plants, while some saprotrophs are destructive to timber, textiles, and other materials. Some fungi form associations with other organisms, most notably with algae to form lichens.

As used herein, the term "fungicide", "antifungal", or "antimycotic" refers to an antibiotic substance or compound that kills or suppresses the growth, viability, or pathogenicity of at least one fungus, fungal cell, fungal tissue or spore.

In the context of this disclosure, "gene" should be understood to refer to a unit of heredity. Each gene is composed of a linear chain of deoxyribonucleotides that can be referred to by the sequence of nucleotides forming the chain. Thus, "sequence" is used to indicate both the ordered listing of the nucleotides that form the chain, and the chain, itself, which has that sequence of nucleotides. "Sequence" is used in the similar way in referring to RNA chains, linear chains made of ribonucleotides. The gene may include regulatory and control sequences, sequences that can be transcribed into an RNA molecule, and may contain sequences with unknown function. The majority of the RNA transcription products are messenger RNAs (mRNAs), which include sequences that are translated into polypeptides and may include sequences that are not translated. It should be recognized that small differences in nucleotide sequence for the same gene can exist between different fungal strains, or even within a particular fungal strain, without altering the identity of the gene.

As used in this disclosure, the terms "growth" or "cell growth" of an organism refer to an increase in mass, density, or number of cells of the organism. Some common methods for the measurement of growth include the determination of the optical density of a cell suspension, the counting of the number of cells in a fixed volume, the counting of the number of cells by measurement of cell division, the measurement of cellular mass or cellular volume, and the like.

As used in this disclosure, the term "growth conditional phenotype" indicates that a fungal strain having such a phenotype exhibits a significantly greater difference in

growth rates in response to a change in one or more of the culture parameters than an otherwise similar strain not having a growth conditional phenotype. Typically, a growth conditional phenotype is described with respect to a single growth culture parameter, such as temperature. Thus, a temperature (or heat-sensitive) mutant (*i.e.*, a fungal strain having a heat-sensitive phenotype) exhibits significantly different growth, and preferably no growth, under non-permissive temperature conditions as compared to growth under permissive conditions. In addition, such mutants preferably also show intermediate growth rates at intermediate, or semi-permissive, temperatures. Similar responses also result from the appropriate growth changes for other types of growth conditional phenotypes.

As used herein, the term "heterologous PBG " means either a nucleic acid encoding a polypeptide or a polypeptide, wherein the polypeptide has at least 42%, 43%, 44%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity or each integer unit of sequence identity from 42-100% in ascending order to *M. grisea* PBG protein (SEQ ID NO:3) and at least 10%, 25%, 50%, 75%, 80%, 90%, 95%, or 99% activity or each integer unit of activity from 10-100% in ascending order of the activity of *M. grisea* PBG protein (SEQ ID NO:3). Examples of heterologous PBG's include, but are not limited to, PBG from *Saccharomyces cerevisiae* (Genbank Accession No. P28789) and PBG from *Candida albicans* (Genbank Accession No. O94048).

As used herein, the term "His-Tag" refers to an encoded polypeptide consisting of multiple consecutive histidine amino acids.

As used herein, the terms "hph," "hygromycin B phosphotransferase," and "hygromycin resistance gene" refer to a hygromycin phosphotransferase gene or gene product.

As used herein, the term "imperfect state" refers to a classification of a fungal organism having no demonstrable sexual life stage.

The term "inhibitor," as used herein, refers to a chemical substance that inactivates the enzymatic activity of PBG or substantially reduces the level of enzymatic activity, wherein "substantially" means a reduction at least as great as the standard deviation for a measurement, preferably a reduction by 50%, more preferably a reduction

of at least one magnitude, i.e. to 10%. The inhibitor may function by interacting directly with the enzyme, a cofactor of the enzyme, the substrate of the enzyme, or any combination thereof.

A polynucleotide may be "introduced" into a fungal cell by any means known to those of skill in the art, including transfection, transformation or transduction, transposable element, electroporation, particle bombardment, infection and the like. The introduced polynucleotide may be maintained in the cell stably if it is incorporated into a non-chromosomal autonomous replicon or integrated into the fungal chromosome. Alternatively, the introduced polynucleotide may be present on an extra-chromosomal non-replicating vector and be transiently expressed or transiently active.

As used herein, the term "knockout" or "gene disruption" refers to the creation of organisms carrying a null mutation (a mutation in which there is no active gene product), a partial null mutation or mutations, or an alteration or alterations in gene regulation by interrupting a DNA sequence through insertion of a foreign piece of DNA. Usually the foreign DNA encodes a selectable marker.

As used herein, the term "mutant form" of a gene refers to a gene that has been altered, either naturally or artificially, changing the base sequence of the gene. The change in the base sequence may be of several different types, including changes of one or more bases for different bases, deletions, and/or insertions, such as by a transposon. In contrast, a normal form of a gene (wild-type) is a form commonly found in natural populations of an organism. Commonly a single form of a gene will predominate in natural populations. In general, such a gene is suitable as a normal form of a gene, however, other forms which provide similar functional characteristics may also be used as a normal gene. In particular, a normal form of a gene does not confer a growth conditional phenotype on the strain having that gene, while a mutant form of a gene suitable for use in these methods does provide such a growth conditional phenotype.

As used herein, the term "Ni-NTA" refers to nickel sepharose.

As used herein, a "normal" form of a gene (wild-type) is a form commonly found in natural populations of an organism. Commonly a single form of a gene will predominate in natural populations. In general, such a gene is suitable as a normal form of a gene, however, other forms which provide similar functional characteristics may also

be used as a normal gene. In particular, a normal form of a gene does not confer a growth conditional phenotype on the strain having that gene, while a mutant form of a gene suitable for use in these methods does provide such a growth conditional phenotype.

As used herein, the term "pathogenicity" refers to a capability of causing disease and/or degree of capacity to cause disease. The term is applied to parasitic micro-organisms in relation to their hosts. As used herein, "pathogenicity," "pathogenic," and the like, encompass the general capability of causing disease as well as various mechanisms and structural and/or functional deviations from normal used in the art to describe the causative factors and/or mechanisms, presence, pathology, and/or progress of disease, such as virulence, host recognition, cell wall degradation, toxin production, infection hyphae, penetration peg production, appressorium production, lesion formation, sporulation, and the like.

The "percent (%) sequence identity" between two polynucleotide or two polypeptide sequences is determined according to either the BLAST program (Basic Local Alignment Search Tool; (Altschul, S.F. *et al.*, 215 *J. Mol. Biol.* 403 (1990) (PMID: 2231712)) or using Smith Waterman Alignment (T.F. Smith & M. S. Waterman 147 *J. Mol. Biol.* 195 (1981) (PMID: 7265238)). It is understood that for the purposes of determining sequence identity when comparing a DNA sequence to an RNA sequence, a thymine nucleotide is equivalent to a uracil nucleotide.

By "polypeptide" is meant a chain of at least two amino acids joined by peptide bonds. The chain may be linear, branched, circular or combinations thereof. The polypeptides may contain amino acid analogs and other modifications, including, but not limited to glycosylated or phosphorylated residues.

As used herein, the term "proliferation" is synonymous to the term "growth."

As used herein, the terms "porphobilinogen deaminase (PBG)" and "porphobilinogen deaminase (PBG) polypeptide" refer to an enzyme that catalyzes the reversible interconversion of porphobilinogen and H_2O to hydroxymethylbilane and NH_3. Although the protein and/or the name of the gene that encodes the protein may differ between species, the terms "PBG" and "HEM3 gene product" are intended to encompass any polypeptide that catalyzes the reversible interconversion of porphobilinogen and H_2O to hydroxymethylbilane and NH_3. For example, the phrase "PBG gene" includes the

HEM3 gene from *M. grisea* as well as genes from other organisms that encode a polypeptide that catalyzes the reversible interconversion of porphobilinogen and H_2O to hydroxymethylbilane and NH_3.

As used herein, "semi-permissive conditions" are conditions in which the relevant culture parameter for a particular growth conditional phenotype is intermediate between permissive conditions and non-permissive conditions. Consequently, in semi-permissive conditions an organism having a growth conditional phenotype will exhibit growth rates intermediate between those shown in permissive conditions and non-permissive conditions. In general, such intermediate growth rate may be due to a mutant cellular component that is partially functional under semi-permissive conditions, essentially fully functional under permissive conditions, and is non-functional or has very low function under non-permissive conditions, where the level of function of that component is related to the growth rate of the organism. An intermediate growth rate may also be a result of a nutrient substance or substances that are present in amounts not sufficient for optimal growth rates to be achieved.

"Sensitivity phenotype" refers to a phenotype that exhibits either hypersensitivity or hyposensitivity.

The term "specific binding" refers to an interaction between PBG and a molecule or compound, wherein the interaction is dependent upon the primary amino acid sequence and/or the tertiary conformation of PBG. A "PBG ligand" is an example of specific binding.

"Transform," as used herein, refers to the introduction of a polynucleotide (single or double stranded DNA, RNA, or a combination thereof) into a living cell by any means. Transformation may be accomplished by a variety of methods, including, but not limited to, electroporation, polyethylene glycol mediated uptake, particle bombardment, agrotransformation, and the like. This process may result in transient or stable expression of the transformed polynucleotide. By "stably transformed" is meant that the sequence of interest is integrated into a replicon in the cell, such as a chromosome or episome. Transformed cells encompass not only the end product of a transformation process, but also the progeny thereof, which retain the polynucleotide of interest.

For the purposes of the invention, "transgenic" refers to any cell, spore, tissue or part, which contains all or part of at least one recombinant polynucleotide. In many cases, all or part of the recombinant polynucleotide is stably integrated into a chromosome or stable extra-chromosomal element, so that it is passed on to successive generations.

As used herein, the term "Tween 20" means sorbitan mono-9-octadecenoate poly(oxy-1,1-ethanediyl).

As used in this disclosure, the term "viability" of an organism refers to the ability of an organism to demonstrate growth under conditions appropriate for the organism, or to demonstrate an active cellular function. Some examples of active cellular functions include respiration as measured by gas evolution, secretion of proteins and/or other compounds, dye exclusion, mobility, dye oxidation, dye reduction, pigment production, changes in medium acidity, and the like.

The present inventors have discovered that disruption of the PBG gene and/or gene product reduces the pathogenicity of *Magnaporthe grisea*. Thus, the inventors are the first to demonstrate that PBG is a target for antibiotics, preferably antifungals.

Accordingly, the invention provides methods for identifying compounds that inhibit PBG gene expression or biological activity of its gene product(s). Such methods include ligand-binding assays, assays for enzyme activity, cell-based assays, and assays for PBG gene expression. The compounds identified by the methods of the invention are useful as antibiotics.

Thus, in one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising contacting a PBG polypeptide with a test compound and detecting the presence or absence of binding between the test compound and the PBG polypeptide, wherein binding indicates that the test compound is a candidate for an antibiotic.

The PBG polypeptides of the invention have the amino acid sequence of a naturally occurring PBG found in a fungus, animal, plant, or microorganism, or have an amino acid sequence derived from a naturally occurring sequence. Preferably the PBG is a fungal PBG. A cDNA encoding *M. grisea* PBG protein is set forth in SEQ ID NO:1, an *M. grisea* PBG genomic DNA is set forth in SEQ ID NO:2, and an *M. grisea* PBG

polypeptide is set forth in SEQ ID NO:3. In one embodiment, the PBG is a *Magnaporthe* PBG. *Magnaporthe* species include, but are not limited to, *Magnaporthe rhizophila, Magnaporthe salvinii, Magnaporthe grisea, Magnaportha oryzae* and *Magnaporthe poae* and the imperfect states of *Magnaporthe* in the genus *Pyricularia*. Preferably, the *Magnaporthe* PBG is from *Magnaporthe grisea*.

In one embodiment, the invention provides a polypeptide consisting essentially of SEQ ID NO:3. For the purposes of the present invention, a polypeptide consisting essentially of SEQ ID NO:3 has at least 90% sequence identity with *M. grisea* PBG (SEQ ID NO:3) and at least 10% of the activity of SEQ ID NO:3. A polypeptide consisting essentially of SEQ ID NO:3 has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity with SEQ ID NO:3 and at least 25%, 50%, 75%, or 90% of the activity of *M. grisea* PBG. Examples of polypeptides consisting essentially of SEQ ID NO:3 include, but are not limited to, polypeptides having the amino acid sequence of SEQ ID NO:3 with the exception that one or more of the amino acids are substituted with structurally similar amino acids providing a "conservative amino acid substitution." Conservative amino acid substitutions are well known to those of skill in the art. Examples of polypeptides consisting essentially of SEQ ID NO:3 include polypeptides having 1, 2, or 3 conservative amino acid substitutions relative to SEQ ID NO:3. Other examples of polypeptides consisting essentially of SEQ ID NO:3 include polypeptides having the sequence of SEQ ID NO:3, but with truncations at either or both the 3' and the 5' end. For example, polypeptides consisting essentially of SEQ ID NO:3 include polypeptides having 1, 2, or 3 amino acids residues removed from either or both 3' and 5' ends relative to SEQ ID NO:3.

In various embodiments, the PBG can be from Powdery Scab (*Spongospora subterranea*), Grey Mould (*Botrytis cinerea*), White Rot (*Armillaria mellea*), Heartrot Fungus (*Ganoderma adspersum*), Brown-Rot (*Piptoporus betulinus*), Corn Smut (*Ustilago maydis*), Heartrot (*Polyporus squamosus*), Gray Leaf Spot (*Cercospora zeae-maydis*), Honey Fungus (*Armillaria gallica*), Root rot (*Armillaria luteobubalina*), Shoestring Rot (*Armillaria ostoyae*), Banana Anthracnose Fungus (*Colletotrichum musae*), Apple-rotting Fungus (*Monilinia fructigena*), Apple-rotting Fungus (*Penicillium expansum*), Clubroot Disease (*Plasmodiophora brassicae*), Potato Blight (*Phytophthora*

infestans), Root pathogen (*Heterobasidion annosum*), Take-all Fungus (*Gaeumannomyces graminis*), Dutch Elm Disease (*Ophiostoma ulmi*), Bean Rust (*Uromyces appendiculatus*), Northern Leaf Spot (*Cochliobolus carbonum*), Milo Disease (*Periconia circinata*), Southern Corn Blight (*Cochliobolus heterostrophus*), Leaf Spot (*Cochliobolus lunata*), Brown Stripe (*Cochliobolus stenospilus*), Panama disease (*Fusarium oxysporum*), Wheat Head Scab Fungus (*Fusarium graminearum*), Cereal Foot Rot (*Fusarium culmorum*), Potato Black Scurf (*Rhizoctonia solani*), Wheat Black Stem Rust (*Puccinia graminis*), White mold (*Sclerotinia sclerotiorum*), and the like.

Fragments of a PBG polypeptide are useful in the methods of the invention. In one embodiment, the PBG fragments include an intact or nearly intact epitope that occurs on the biologically active wild-type PBG. The fragments comprise at least 10 consecutive amino acids of a PBG. The fragments comprises at least 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 225, 250, 300, 325 or at least 338 consecutive amino acids residues of a PBG. In one embodiment, the fragment is from a *Magnaporthe* PBG. In one embodiment, the fragment contains an amino acid sequence conserved among fungal PBG's.

Polypeptides having at least 42% sequence identity with *M. grisea* PBG (SEQ ID NO:3) protein are also useful in the methods of the invention. In one embodiment, the sequence identity is at least 42%, 43%, 44%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, or any integer from 42-100% sequence identity in ascending order with *M. grisea* PBG (SEQ ID NO:3) protein. In addition, it is preferred that polypeptides of the invention have at least 10% of the activity of *M. grisea* PBG (SEQ ID NO:3) protein. PBG polypeptides of the invention have at least 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or at least 90% of the activity of *M. grisea* PBG (SEQ ID NO:3) protein.

Thus, in another embodiment, the invention provides a method for identifying a test compound as a candidate for a fungicide, comprising: contacting a test compound with at least one polypeptide selected from the group consisting of: a polypeptide consisting essentially of SEQ ID NO:3, a polypeptide having at least ten consecutive amino acids of an *M. grisea* PBG (SEQ ID NO:3) protein, a polypeptide having at least 42% sequence identity with an *M. grisea* PBG (SEQ ID NO:3) protein and at least 10%

of the activity of an *M. grisea* PBG (SEQ ID NO:3) protein; and a polypeptide consisting of at least 50 amino acids having at least 42% sequence identity with an *M. grisea* PBG (SEQ ID NO:3) protein and at least 10% of the activity of an *M. grisea* PBG (SEQ ID NO:3) protein; and detecting the presence and/or absence of binding between the test compound and the polypeptide, wherein binding indicates that the test compound is a candidate for an antibiotic.

Any technique for detecting the binding of a ligand to its target may be used in the methods of the invention. For example, the ligand and target are combined in a buffer. Many methods for detecting the binding of a ligand to its target are known in the art, and include, but are not limited to, the detection of an immobilized ligand-target complex or the detection of a change in the properties of a target when it is bound to a ligand. For example, in one embodiment, an array of immobilized candidate ligands is provided. The immobilized ligands are contacted with a PBG protein or a fragment or variant thereof, the unbound protein is removed and the bound PBG is detected. In a preferred embodiment, bound PBG is detected using a labeled binding partner, such as a labeled antibody. In an alternate preferred embodiment, PBG is labeled prior to contacting the immobilized candidate ligands. Preferred labels include fluorescent or radioactive moieties. Preferred detection methods include fluorescence correlation spectroscopy (FCS) and FCS-related confocal nanofluorimetric methods.

Once a compound is identified as a candidate for an antibiotic, it can be tested for the ability to inhibit PBG enzymatic activity. The compounds can be tested using either *in vitro* or cell based assays. Alternatively, a compound can be tested by applying it directly to a fungus or fungal cell, or expressing it therein, and monitoring the fungus or fungal cell for changes or decreases in growth, development, viability, pathogenicity, or alterations in gene expression. Thus, in one embodiment, the invention provides a method for determining whether a compound identified as an antibiotic candidate by an above method has antifungal activity, further comprising: contacting a fungus or fungal cells with the antifungal candidate and detecting a decrease in the growth, viability, or pathogenicity of the fungus or fungal cells.

By decrease in growth, is meant that the antifungal candidate causes at least a 10% decrease in the growth of the fungus or fungal cells, as compared to the growth of

the fungus or fungal cells in the absence of the antifungal candidate. By a decrease in viability is meant that at least 20% of the fungal cells, or portion of the fungus contacted with the antifungal candidate are nonviable. Preferably, the growth or viability will be decreased by at least 40%. More preferably, the growth or viability will be decreased by at least 50%, 75% or at least 90% or more. Methods for measuring fungal growth and cell viability are known to those skilled in the art. By decrease in pathogenicity, is meant that the antifungal candidate causes at least a 10% decrease in the disease caused by contact of the fungal pathogen with its host, as compared to the disease caused in the absence of the antifungal candidate. Preferably, the disease will be decreased by at least 40%. More preferably, the disease will be decreased by at least 50%, 75% or at least 90% or more. Methods for measuring fungal disease are well known to those skilled in the art, and include such metrics as lesion formation, lesion size, sporulation, respiratory failure, and/or death.

The ability of a compound to inhibit PBG activity can be detected using *in vitro* enzymatic assays in which the disappearance of a substrate or the appearance of a product is directly or indirectly detected. PBG catalyzes the reversible interconversion of porphobilinogen and H_2O to hydroxymethylbilane and NH_3 (see Figure 1). Methods for measuring the progression of the PBG enzymatic reaction and/or a change in the concentration of the individual reactants porphobilinogen, H_2O, hydroxymethylbilane, and/or NH_3, include spectrophotometry, fluorimetry, mass spectroscopy, thin layer chromatography (TLC) and reverse phase HPLC.

Thus, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising: contacting porphobilinogen and H_2O with a PBG in the presence and absence of a test compound or contacting hydroxymethylbilane and NH_3 with a PBG in the presence and absence of a test compound; and determining a change in concentration for at least one of porphobilinogen, H_2O, hydroxymethylbilane and/or NH_3 in the presence and absence of the test compound, wherein a change in the concentration for any of the above reactants indicates that the test compound is a candidate for an antibiotic.

Enzymatically active fragments of *M. grisea* PBG set forth in SEQ ID NO:3 are also useful in the methods of the invention. For example, an enzymatically active

polypeptide comprising at least 50 consecutive amino acid residues and at least 10% of the activity of *M. grisea* PBG set forth in SEQ ID NO:3 are useful in the methods of the invention. In addition, enzymatically active polypeptides having at least 10% of the activity of SEQ ID NO:3 and at least 42%, 43%, 44%, 45%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity with SEQ ID NO:3 are useful in the methods of the invention. Most preferably, the enzymatically active polypeptide has at least 42% sequence identity with SEQ ID NO:3 and at least 25%, 75% or at least 90% of the activity thereof.

Thus, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising: contacting porphobilinogen and H_2O or hydroxymethylbilane and NH_3 with a polypeptide selected from the group consisting of: a polypeptide consisting essentially of SEQ ID NO:3, a polypeptide having at least 42% sequence identity with the *M. grisea* PBG set forth in SEQ ID NO:3 and having at least 10% of the activity thereof, a polypeptide comprising at least 50 consecutive amino acids of *M. grisea* PBG set forth in SEQ ID NO:3 and having at least 10% of the activity thereof, and a polypeptide consisting of at least 50 amino acids and having at least 42% sequence identity with *M. grisea* PBG set forth in SEQ ID NO:3 and having at least 10% of the activity thereof; contacting porphobilinogen and H_2O or hydroxymethylbilane and NH_3 with the polypeptide and a test compound; and determining a change in concentration for at least one of porphobilinogen, H_2O, hydroxymethylbilane and/or NH_3 in the presence and absence of the test compound, wherein a change in concentration for any of the above substances indicates that the test compound is a candidate for an antibiotic. For the *in vitro* enzymatic assays, PBG protein and derivatives thereof may be purified from a fungus or may be recombinantly produced in and purified from an archael, bacterial, fungal, or other eukaryotic cell culture. Preferably these proteins are produced using an *E. coli*, yeast, or filamentous fungal expression system. Such methods for the production of polypeptides are known to those skilled in the art. Examples of methods for the measurement of PBG enzymatic activity are described in Kurtz & Marrinan, 217 *Mol. Gen. Genet.* 47-52 (1989); Correa et al., 48 *Enzyme Protein* 275-81 (1994); Vazquez-Prado et al., 18 *J. Inherit. Metab. Dis.* 66-71 (1995); and Anderson & Desnick, 28 *Enzyme* 146-57 (1982).

As an alternative to *in vitro* assays, the invention also provides cell-based assays. In one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising: a) measuring the expression or activity of a PBG in a cell, cells, tissue, or an organism in the absence of a test compound; b) contacting the cell, cells, tissue, or organism with the test compound and measuring the expression or activity of the PBG in the cell, cells, tissue, or organism; and c) comparing the expression or activity of the PBG in steps (a) and (b), wherein an altered expression or activity in the presence of the test compound indicates that the compound is a candidate for an antibiotic.

Expression of PBG can be measured by detecting the PBG primary transcript or mRNA, PBG polypeptide, or PBG enzymatic activity. Methods for detecting the expression of RNA and proteins are known to those skilled in the art. (See, *e.g.*, *Current Protocols in Molecular Biology*, Ausubel *et al.*, eds., Greene Publishing & Wiley-Interscience, New York, (1995)). The method of detection is not critical to the present invention. Methods for detecting PBG RNA include, but are not limited to amplification assays such as quantitative reverse transcriptase-PCR, and/or hybridization assays such as Northern analysis, dot blots, slot blots, in-situ hybridization, transcriptional fusions using a PBG promoter fused to a reporter gene, DNA assays, and microarray assays.

Methods for detecting protein expression include, but are not limited to, immunodetection methods such as Western blots, ELISA assays, polyacrylamide gel electrophoresis, mass spectroscopy, and enzymatic assays. Also, any reporter gene system may be used to detect PBG protein expression. For detection using gene reporter systems, a polynucleotide encoding a reporter protein is fused in frame with PBG, so as to produce a chimeric polypeptide. Methods for using reporter systems are known to those skilled in the art.

Chemicals, compounds or compositions identified by the above methods as modulators of PBG expression or activity can then be used to control fungal growth. Diseases such as rusts, mildews, and blights spread rapidly once established. Fungicides are thus routinely applied to growing and stored crops as a preventive measure, generally as foliar sprays or seed dressings. For example, compounds that inhibit fungal growth can be applied to a fungus or expressed in a fungus, in order to prevent fungal growth.

Thus, the invention provides a method for inhibiting fungal growth, comprising contacting a fungus with a compound identified by the methods of the invention as having antifungal activity.

Antifungals and antifungal inhibitor candidates identified by the methods of the invention can be used to control the growth of undesired fungi, including ascomycota, zygomycota, basidiomycota, chytridiomycota, and lichens. Examples of undesired fungi include, but are not limited to Powdery Scab (*Spongospora subterranea*), Grey Mould (*Botrytis cinerea*), White Rot (*Armillaria mellea*), Heartrot Fungus (*Ganoderma adspersum*), Brown-Rot (*Piptoporus betulinus*), Corn Smut (*Ustilago maydis*), Heartrot (*Polyporus squamosus*), Gray Leaf Spot (*Cercospora zeae-maydis*), Honey Fungus (*Armillaria gallica*), Root rot (*Armillaria luteobubalina*), Shoestring Rot (*Armillaria ostoyae*), Banana Anthracnose Fungus (*Colletotrichum musae*), Apple-rotting Fungus (*Monilinia fructigena*), Apple-rotting Fungus (*Penicillium expansum*), Clubroot Disease (*Plasmodiophora brassicae*), Potato Blight (*Phytophthora infestans*), Root pathogen (*Heterobasidion annosum*), Take-all Fungus (*Gaeumannomyces graminis*), Dutch Elm Disease (*Ophiostoma ulmi*), Bean Rust (*Uromyces appendiculatus*), Northern Leaf Spot (*Cochliobolus carbonum*), Milo Disease (*Periconia circinata*), Southern Corn Blight (*Cochliobolus heterostrophus*), Leaf Spot (*Cochliobolus lunata*), Brown Stripe (*Cochliobolus stenospilus*), Panama disease (*Fusarium oxysporum*), Wheat Head Scab Fungus (*Fusarium graminearum*), Cereal Foot Rot (*Fusarium culmorum*), Potato Black Scurf (*Rhizoctonia solani*), Wheat Black Stem Rust (*Puccinia graminis*), White mold (*Sclerotinia sclerotiorum*), diseases of animals such as infections of lungs, blood, brain, skin, scalp, nails or other tissues (*Aspergillus fumigatus Aspergillus* sp. *Fusraium* sp., *Trichophyton* sp., *Epidermophyton* sp., and *Microsporum* sp., and the like).

Also provided in the invention are methods of screening for an antibiotic by determining the *in vivo* activity of a test compound against two separate fungal organisms, wherein the fungal organisms comprise a first form of a PBG and a second form of the PBG, respectively. In the methods of the invention, at least one of the two forms of the PBG has at least 10% of the activity of the polypeptide set forth in SEQ ID NO:3. The methods comprise comparing the growth of the two organisms in the presence of the test compound relative to their respective controls without test compound.

A difference in growth between the two organisms in the presence of the test compound indicates that the test compound is a candidate for an antibiotic.

The forms of a PBG useful in the methods of the invention are selected from the group consisting of: a nucleic acid encoding SEQ ID NO:3, a nucleic acid encoding a polypeptide consisting essentially of SEQ ID NO:3, SEQ ID NO:1 or SEQ ID NO:2, SEQ ID NO:1 or SEQ ID NO:2 comprising a mutation either reducing or abolishing PBG protein activity, a heterologous PBG, and a heterologous PBG comprising a mutation either reducing or abolishing PBG protein activity. Any combination of two different forms of the PBG genes listed above are useful in the methods of the invention, with the limitation that at least one of the forms of the PBG has at least 10% of the activity of the polypeptide set forth in SEQ ID NO:3.

Thus, in one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising: providing an organism having a first form of a PBG; providing an organism having a second form of the PBG; and determining the growth of the organism having the first form of the PBG and the growth of the organism having the second form of the PBG in the presence of the test compound, wherein a difference in growth between the two organisms in the presence of the test compound indicates that the test compound is a candidate for an antibiotic. It is recognized in the art that the optional determination of the growth of the organism having the first form of the PBG and the growth of the organism having the second form of the PBG in the absence of any test compounds is performed to control for any inherent differences in growth as a result of the different genes. Growth and/or proliferation of an organism are measured by methods well known in the art such as optical density measurements, and the like. In a preferred embodiment, the organism is *Magnaporthe grisea*.

In another embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising: providing an organism having a first form of a PBG; providing a comparison organism having a second form of the PBG; and determining the pathogenicity of the organism having the first form of the PBG and the organism having the second form of the PBG in the presence of the test compound, wherein a difference in pathogenicity between the two organisms in the presence of the

test compound indicates that the test compound is a candidate for an antibiotic. In an optional embodiment of the inventon, the pathogenicity of the organism having the first form of the PBG and the organism having the second form of the PBG in the absence of any test compounds is determined to control for any inherent differences in pathogenicity as a result of the different genes. Pathogenicity of an organism is measured by methods well known in the art such as lesion number, lesion size, sporulation, and the like. In a preferred embodiment the organism is *Magnaporthe grisea.*

In one embodiment of the invention, the first form of a PBG is SEQ ID NO:1 or SEQ ID NO:2, and the second form of the PBG is a PBG that confers a growth conditional phenotype (*i.e.* a heme requiring phenotype) and/or a hypersensitivity or hyposensitivity phenotype on the organism. In a related embodiment of the invention, the second form of the PBG is SEQ ID NO:1 comprising a transposon insertion that reduces activity. In a related embodiment of the invention, the second form of a PBG is SEQ ID NO:1 comprising a transposon insertion that abolishes activity. In a related embodiment of the invention, the second form of the PBG is SEQ ID NO:2 comprising a transposon insertion that reduces activity. In a related embodiment of the invention, the second form of the PBG is SEQ ID NO:2 comprising a transposon insertion that abolishes activity. In a related embodiment of the invention, the second form of the PBG is *S. cerevisiae* PBG. In a related embodiment of the invention, the second form of the PBG is *C. albicans* PBG.

In another embodiment of the invention, the first form of a PBG is *S. cerevisiae* PBG and the second form of the PBG is *S. cerevisiae* PBG comprising a transposon insertion that reduces activity. In a related embodiment of the invention, the second form of the PBG is *S. cerevisiae* PBG comprising a transposon insertion that abolishes activity. In another embodiment of the invention, the first form of a PBG *C. albicans* PBG and the second form of the PBG is *C. albicans* PBG comprising a transposon insertion that reduces activity. In a related embodiment of the invention, the second form of the PBG is *C. albicans* PBG comprising a transposon insertion that abolishes activity.

Conditional lethal mutants and/or antipathogenic mutants identify particular biochemical and/or genetic pathways given that at least one identified target gene is present in that pathway. Knowledge of these pathways allows for the screening of test

compounds as candidates for antibiotics as inhibitors of the substrates, products, proteins and/or enzymes of the pathway. The invention provides methods of screening for an antibiotic by determining whether a test compound is active against the heme biosynthetic pathway on which PBG functions. Pathways known in the art are found at the Kyoto Encyclopedia of Genes and Genomes and in standard biochemistry texts (See, *e.g.* Lehninger *et al.*, Principles of Biochemistry, New York, Worth Publishers (1993)).

Thus, in one embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which PBG functions, comprising: providing an organism having a first form of a gene in the heme biosynthetic pathway; providing an organism having a second form of the gene in the heme biosynthetic pathway; and determining the growth of the two organisms in the presence of a test compound, wherein a difference in growth between the organism having the first form of the gene and the organism having the second form of the gene in the presence of the test compound indicates that the test compound is a candidate for an antibiotic. It is recognized in the art that the optional determination of the growth of the organism having the first form of the gene and the organism having the second form of the gene in the absence of any test compounds is performed to control for any inherent differences in growth as a result of the different genes. Growth and/or proliferation of an organism are measured by methods well known in the art such as optical density measurements, and the like. In a preferred embodiment, the organism is *Magnaporthe grisea*.

The forms of a gene in the heme biosynthetic pathway useful in the methods of the invention include, for example, wild-type and mutated genes encoding 5-porphobilinogen synthase or uroporphyrinogen-III synthase from any organism, preferably from a fungal organism, and most preferrably from *M. grisea*. The forms of a mutated gene in the heme biosynthetic pathway comprise a mutation either reducing or abolishing protein activity. In one example, the form of a gene in the heme biosynthetic pathway comprises a transposon insertion. Any combination of a first form of a gene in the heme biosynthetic pathway and a second form of the gene listed above are useful in the methods of the invention, with the limitation that one of the forms of a gene in the

heme biosynthetic pathway has at least 10% of the activity of the corresponding *M. grisea* gene.

In another embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which PBG functions, comprising: providing an organism having a first form of a gene in the heme biosynthetic pathway; providing an organism having a second form of the gene in the heme biosynthetic pathway; and determining the pathogenicity of the two organisms in the presence of the test compound, wherein a difference in pathogenicity between the organism having the first form of the gene and the organism having the second form of the gene in the presence of the test compound indicates that the test compound is a candidate for an antibiotic. In an optional embodiment of the inventon, the pathogenicity of the two organisms in the absence of any test compounds is determined to control for any inherent differences in pathogenicity as a result of the different genes. Pathogenicity of an organism is measured by methods well known in the art such as lesion number, lesion size, sporulation, and the like. In a preferred embodiment the organism is *Magnaporthe grisea*.

Thus, in an alternate embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which PBG functions, comprising: providing paired growth media containing a test compound, wherein the paired growth media comprise a first medium and a second medium and the second medium contains a higher level of heme than the first medium; inoculating the first and the second medium with an organism; and determining the growth of the organism, wherein a difference in growth of the organism between the first and the second medium indicates that the test compound is a candidate for an antibiotic. In one embodiment of the invention, the growth of the organism is determined in the first and the second medium in the absence of any test compounds to control for any inherent differences in growth as a result of the different media. Growth and/or proliferation of the organism are measured by methods well known in the art such as optical density measurements, and the like. In a preferred embodiment, the organism is *Magnaporthe grisea*.

One embodiment of the invention is directed to the use of multi-well plates for screening of antibiotic compounds. The use of multi-well plates is a format that readily accommodates multiple different assays to characterize various compounds, concentrations of compounds, and fungal organisms in varying combinations and formats. Certain testing parameters for the screening method can significantly affect the identification of growth inhibitors, and thus can be manipulated to optimize screening efficiency and/or reliability. Notable among these factors are variable sensitivities of different mutants, increasing hypersensitivity with increasingly less permissive conditions, an apparent increase in hypersensitivity with increasing compound concentration, and other factors known to those in the art.

EXPERIMENTAL

Example 1

Construction of Plasmids with a Transposon Containing a Selectable Marker

Construction of Sif transposon:

Sif was constructed using the GPS3 vector from the GPS-M mutagenesis system from New England Biolabs, Inc. (Beverly, MA) as a backbone. This system is based on the bacterial transposon Tn7. The following manipulations were done to GPS3 according to Sambrook *et al.*, Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press (1989). The kanamycin resistance gene (npt) contained between the Tn7 arms was removed by EcoRV digestion. The bacterial hygromycin B phosphotransferase (hph) gene (Gritz & Davies, 25 *Gene* 179 (1983) (PMID: 6319235)) under control of the *Aspergillus nidulans* trpC promoter and terminator (Mullaney *et al.*, 199 *Mol. Gen. Genet.* 37 (1985) (PMID: 3158796)) was cloned by a HpaI/EcoRV blunt ligation into the Tn7 arms of the GPS3 vector yielding pSif1. Excision of the ampicillin resistance gene (bla) from pSif1 was achieved by cutting pSif1 with XmnI and BglI followed by a T4 DNA polymerase treatment to remove the 3' overhangs left by the BglI digestion and religation of the plasmid to yield pSif. Top 10F' electrocompetent *E. coli* cells (Invitrogen) were transformed with ligation mixture according to manufacturer's recommendations. Transformants containing the Sif transposon were selected on LB

agar (Sambrook *et al.*, *supra*) containing 50μg/ml of hygromycin B (Sigma Chem. Co., St. Louis, MO).

Example 2

Construction of a Fungal Cosmid Library

Cosmid libraries were constructed in the pcosKA5 vector (Hamer *et al.*, 98 *Proc. Nat'l. Acad. Sci. USA* 5110 (2001) (PMID: 11296265)) as described in Sambrook *et al.* Cosmid libraries were quality checked by pulsed-field gel electrophoresis, restriction digestion analysis, and PCR identification of single genes.

Example 3

Construction of Cosmids with Transposon Insertion into Fungal Genes

Sif Transposition into a Cosmid:

Transposition of Sif into the cosmid framework was carried out as described by the GPS-M mutagenesis system (New England Biolabs, Inc.). Briefly, 2μl of the 10X GPS buffer, 70ng of supercoiled pSIF, 8-12μg of target cosmid DNA were mixed and taken to a final volume of 20μl with water. 1μl of transposase (TnsABC) was added to the reaction and incubated for 10 minutes at 37°C to allow the assembly reaction to occur. After the assembly reaction, 1μl of start solution was added to the tube, mixed well, and incubated for 1 hour at 37°C followed by heat inactivation of the proteins at 75°C for 10 minutes. Destruction of the remaining untransposed pSif was completed by PISceI digestion at 37°C for 2 hours followed by a 10 minute incubation at 75°C to inactivate the proteins. Transformation of Top10F' electrocompetent cells (Invitrogen) was done according to manufacturers recommendations. Sif-containing cosmid transformants were selected by growth on LB agar plates containing 50μg/ml of hygromycin B (Sigma Chem. Co.) and 100μg/ml of Ampicillin (Sigma Chem. Co.).

Example 4

High Throughput Preparation and Verification of Transposon Insertion Into the *M. grisea* HEM3 Gene

E. coli strains containing cosmids with transposon insertions were picked to 96 well growth blocks (Beckman Co.) containing 1.5ml of TB (Terrific Broth, Sambrook *et al.*, *supra*) supplemented with 50μg/ml of ampicillin. Blocks were incubated with shaking at 37°C overnight. *E. coli* cells were pelleted by centrifugation and cosmids were isolated by a modified alkaline lysis method (Marra *et al.*, 7 *Genome Res.* 1072 (1997) (PMID: 9371743)). DNA quality was checked by electrophoresis on agarose gels. Cosmids were sequenced using primers from the ends of each transposon and commercial dideoxy sequencing kits (Big Dye Terminators, Perkin Elmer Co.). Sequencing reactions were analyzed on an ABI377 DNA sequencer (Perkin Elmer Co.).

The DNA sequences adjacent to the site of the transposon insertion were used to search DNA and protein databases using the BLAST algorithms (Altschul *et al.*, *supra*). A single insertion of SIF into the *Magnaporthe grisea* HEM3 gene was chosen for further analysis. This construct was designated cpgmra0012033e10 and it contains the SIF transposon insertion in the coding region approximately between amino acids 131 and 168.

Example 5

Preparation of HEM3 Cosmid DNA and Transformation of *Magnaporthe grisea*

Cosmid DNA from the HEM3 transposon tagged cosmid clone was prepared using QIAGEN Plasmid Maxi Kit (Qiagen), and digested by PI-PspI (New England Biolabs, Inc.). Fungal electro-transformation was performed essentially as described (Wu *et al.*, 10 *MPMI* 700 (1997)). Briefly, *M. grisea* strain Guy 11 was grown in complete liquid media (Talbot *et al.*, 5 *Plant Cell* 1575 (1993) (PMID: 8312740)) shaking at 120rpm for 3 days at 25°C in the dark. Mycelia was harvested and washed with sterile H_2O and digested with 4mg/ml beta-glucanase (InterSpex) for 4-6 hours to generate protoplasts. Protoplasts were collected by centrifugation and resuspended in 20% sucrose at a concentration of $2x10^8$ protoplasts/ml. 50μl of protoplast suspension was mixed with 10-20μg of the cosmid DNA and pulsed using a Gene Pulser II instrument (BioRad) set with the following parameters: 200ohm, 25μF, and 0.6kV. Transformed protoplasts were regenerated in complete agar media (Talbot *et al.*, *supra*) with the addition of 20% sucrose for one day, then overlayed with CM agar media

containing hygromycin B (250μg/ml) to select transformants. Transformants were screened for homologous recombination events in the target gene by PCR (Hamer *et al.*, *supra*). Two independent strains were identified and are hereby referred to as K1-6 and K1-23, respectively.

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Example 6

Effect of Transposon Insertion on *Magnaporthe* pathogenicity

The target fungal strains, K1-6 and K1-23, obtained in Example 5 and the wild-type strain, Guy11, were subjected to a pathogenicity assay to observe infection over a 1-
10 week period. Rice infection assays were performed using India rice cultivar CO39 essentially as described in Valent *et al.* (Valent *et al.*, 127 *Genetics* 87 (1991) (PMID: 2016048)). All three strains were grown for spore production on complete agar media. Spores were harvested and the concentration of spores adjusted for whole plant inoculations. Two-week-old seedlings of cultivar CO39 were sprayed with 12ml of
15 conidial suspension (5×10^4 conidia per ml in 0.01% Tween-20 solution). The inoculated plants were incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27°C 12 hours/21°C 12 hours at 70% humidity) for an additional 5.5 days. Leaf samples were taken at 3, 5, and 7 days post-inoculation and examined for signs of successful infection (*i.e.* lesions). Figure 2 shows the effects of HEM3 gene
20 disruption on *Magnaporthe* infection at five days post-inoculation.

Example 7

Cloning, Expression, and Purification of Porphobilinogen Deaminase

The following is a protocol to obtain a purified porphobilinogen deaminase
25 protein.

Cloning and expression strategies:

A PBG encoding nucleic acid is cloned into *E. coli* (pET vectors-Novagen), Baculovirus (Pharmingen) and Yeast (Invitrogen) expression vectors containing His/fusion protein tags, and the expression of recombinant protein is evaluated by SDS-
30 PAGE and Western blot analysis.

Extraction:

Extract recombinant protein from 250ml cell pellet in 3ml of extraction buffer by sonicating 6 times, with 6 second pulses at 4°C. Centrifuge extract at 15000xg for 10 minutes and collect supernatant. Assess biological activity of the recombinant protein by activity assay.

Purification:

Purify recombinant protein by Ni-NTA affinity chromatography (Qiagen). Purification protocol (perform all steps at 4°C):

- Use 3ml Ni-beads
- Equilibrate column with the buffer
- Load protein extract
- Wash with the equilibration buffer
- Elute bound protein with 0.5M imidazole

Example 8

Assays for Measuring Binding of Test Compounds to Porphobilinogen Deaminase

The following is a protocol to identify test compounds that bind to the porphobilinogen deaminase protein.

- Purified full-length PBG polypeptide with a His/fusion protein tag (Example 7) is bound to a HISGRAB Nickel Coated Plate (Pierce, Rockford, IL) following manufacturer's instructions.
- Buffer conditions are optimized (*e.g.* ionic strength or pH, Shoolingin-Jordan *et al.,* 281 *Methods Enzymol.* 309-16 (1997) (PMID: 9250995)) for binding of radiolabeled porphobilinogen, hydroxymethylbilane or NH_3 to the bound porphobilinogen deaminase.
- Screening of test compounds is performed by adding test compound and radioactive porphobilinogen, hydroxymethylbilane or NH_3 to the wells of the HISGRAB plate containing bound porphobilinogen deaminase.
- The wells are washed to remove excess labeled ligand and scintillation fluid (SCINTIVERSE, Fisher Scientific) is added to each well.

- The plates are read in a microplate scintillation counter.
- Candidate compounds are identified as wells with lower radioactivity as compared to control wells with no test compound added.

Additionally, a purified polypeptide comprising 10-50 amino acids from the *M. grisea* porphobilinogen deaminase is screened in the same way. A polypeptide comprising 10-50 amino acids is generated by subcloning a portion of the PBG encoding nucleic acid into a protein expression vector that adds a His-Tag when expressed (see Example 7). Oligonucleotide primers are designed to amplify a portion of the PBG encoding nucleic acid using the polymerase chain reaction amplification method. The DNA fragment encoding a polypeptide of 10-50 amino acids is cloned into an expression vector, expressed in a host organism and purified as described in Example 7 above.

Test compounds that bind PBG are further tested for antibiotic activity. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores are harvested into minimal media to a concentration of 2×10^5 spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100μg/ml. Solvent only is added to the second culture. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The growth curves of the solvent control sample and the test compound sample are compared. A test compound is an antibiotic candidate if the growth of the culture containing the test compound is less than the growth of the control culture.

Test compounds that bind PBG are further tested for antipathogenic activity. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores are harvested into water with 0.01% Tween 20 to a concentration of 5×10^4 spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100μg/ml. Solvent only is added to the second culture. Rice infection assays are performed using India rice cultivar CO39 essentially as described in Valent *et al.*, *supra*). Two-week-old seedlings of cultivar CO39 are sprayed with 12ml of conidial suspension. The inoculated plants are incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27°C 12 hours/21°C 12 hours at 70% humidity) for an additional 5.5 days. Leaf samples are

examined at 5 days post-inoculation to determine the extent of pathogenicity as compared to the control samples.

Alternatively, antipathogenic activity can be assessed using an excised leaf pathogenicity assay. Spore suspensions are prepared in water only to a concentration of $5x10^4$ spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100µg/ml. Solvent only is added to the second culture. Detached leaf assays are performed by excising 1cm segments of rice leaves from India rice cultivar CO39 and placing them on 1% agarose in water. 10µl of each spore suspension is place on the leaf segments and the samples are incubated at 25°C for 5 days in the dark. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity as compared to the control samples.

Example 9

Assays for Testing Inhibitors or Candidates for Inhibition of Porphobilinogen Deaminase Activity

The enzymatic activity of porphobilinogen deaminase is determined in the presence and absence of candidate compounds in a suitable reaction mixture, such as described by Kurtz & Marrinan (1989); Correa et al. (1994); Vazquez-Prado et al. (1995); or Anderson & Desnick (1982), *supra*. Candidate compounds are identified by a decrease in products or a lack of a decrease in substrates in the presence of the compound, with the reaction proceeding in either direction.

Candidate compounds are additionally determined in the same manner using a polypeptide comprising a fragment of the *M. grisea* porphobilinogen deaminase. The PBG polypeptide fragment is generated by subcloning a portion of the PBG encoding nucleic acid into a protein expression vector that adds a His-Tag when expressed (see Example 7). Oligonucleotide primers are designed to amplify a portion of the PBG encoding nucleic acid using polymerase chain reaction amplification method. The DNA fragment encoding the PBG polypeptide fragment is cloned into an expression vector, expressed and purified as described in Example 7 above.

Test compounds identified as inhibitors of PBG activity are further tested for antibiotic activity. *Magnaporthe grisea* fungal cells are grown under standard fungal

growth conditions that are well known and described in the art. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores are harvested into minimal media to a concentration of 2 x 10^5 spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100μg/ml. Solvent only is added to the second culture. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The growth curves of the solvent control sample and the test compound sample are compared. A test compound is an antibiotic candidate if the growth of the culture containing the test compound is less than the growth of the control culture.

Test compounds identified as inhibitors of PBG activity are further tested for antipathogenic activity. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores are harvested into water with 0.01% Tween 20 to a concentration of $5x10^4$ spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100μg/ml. Solvent only is added to the second culture. Rice infection assays are performed using India rice cultivar CO39 essentially as described in Valent *et al.*, *supra*. Two-week-old seedlings of cultivar CO39 are sprayed with 12ml of conidial suspension. The inoculated plants are incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27°C 12 hours/21°C 12 hours at 70% humidity) for an additional 5.5 days. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity as compared to the control samples.

Alternatively, antipathogenic activity is assessed using an excised leaf pathogenicity assay. Spore suspensions are prepared in water only to a concentration of $5x10^4$ spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100μg/ml. Solvent only is added to the second culture. Detached leaf assays are performed by excising 1cm segments of rice leaves from India rice cultivar CO39 and placing them on 1% agarose in water. 10μl of each spore suspension is place on the leaf segments and the samples are incubated at 25°C for 5 days in the dark. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity as compared to the control samples.

Example 10

Assays for Testing Compounds for Alteration of Porphobilinogen Deaminase Gene Expression

Magnaporthe grisea fungal cells are grown under standard fungal growth conditions that are well known and described in the art. Wild-type *M. grisea* spores are harvested from cultures grown on complete agar or oatmeal agar media after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. 25ml cultures are prepared to which test compounds will be added at various concentrations. A culture with no test compound present is included as a control. The cultures are incubated at 25°C for 3 days after which test compound or solvent only control is added. The cultures are incubated an additional 18 hours. Fungal mycelia is harvested by filtration through Miracloth (CalBiochem, La Jolla, CA), washed with water, and frozen in liquid nitrogen. Total RNA is extracted with TRIZOL Reagent using the methods provided by the manufacturer (Life Technologies, Rockville, MD). Expression is analyzed by Northern analysis of the RNA samples as described (Sambrook *et al.*, *supra*) using a radiolabeled fragment of the PBG encoding nucleic acid as a probe. Test compounds resulting in an altered level of PBG mRNA relative to the untreated control sample are identified as candidate antibiotic compounds.

Test compounds identified as inhibitors of PBG expression are further tested for antibiotic activity. *Magnaporthe grisea* fungal cells are grown under standard fungal growth conditions that are well known and described in the art. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores are harvested into minimal media to a concentration of $2x10^5$ spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100μg/ml. Solvent only is added to the second culture. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The growth curves of the solvent control sample and the test compound sample are compared. A test compound is an antibiotic candidate if the growth of the culture containing the test compound is less than the growth of the control culture.

Test compounds identified as inhibitors of PBG gene expression are further tested for antipathogenic activity. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores are harvested into water with 0.01% Tween 20 to a concentration of $5x10^4$ spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100μg/ml. Solvent only is added to the second culture. Rice infection assays are performed using India rice cultivar CO39 essentially as described in Valent *et al.*, *supra*. Two-week-old seedlings of cultivar CO39 are sprayed with 12ml of conidial suspension. The inoculated plants are incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27°C 12 hours/21°C 12 hours at 70% humidity) for an additional 5.5 days. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity as compared to the control samples.

Alternatively, antipathogenic activity is assessed using an excised leaf pathogenicity assay. Spore suspensions are prepared in water only to a concentration of $5x10^4$ spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100μg/ml. Solvent only is added to the second culture. Detached leaf assays are performed by excising 1 cm segments of rice leaves from India rice cultivar CO39 and placing them on 1% agarose in water. 10μl of each spore suspension is place on the leaf segments and the samples are incubated at 25°C for 5 days in the dark. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity as compared to the control samples.

Example 11

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of Porphobilinogen Deaminase that Lacks Activity

The effect of test compounds on the growth of wild-type fungal cells and mutant fungal cells having a mutant PBG gene is measured and compared as follows. *Magnaporthe grisea* fungal cells containing a mutant form of the PBG gene that lacks activity, for example a PBG gene containing a transposon insertion, are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium

containing heme, hemin, or protoporphyrin IX (Sigma) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium containing heme, hemin, or protoporphyrin IX to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores are added to each well of 96-well plates to which a test compound is added (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present (growth control), and wells without cells are included as controls (negative control). The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild-type cells are screened under the same conditions.

The effect of each of the test compounds on the mutant and wild-type fungal cells is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound)/OD_{590} (growth control) x 100. The percent of growth inhibition in the presence of the test compound on the mutant and wild-type fungal strains are compared. Compounds that show differential growth inhibition between the mutant and the wild-type cells are identified as potential antifungal compounds. Similar protocols may be found in Kirsch & DiDomenico, 26 *Biotechnology* 177 (1994) (PMID: 7749303)).

Test compounds that produce a differential growth response between the mutant and wild-type fungal strains are further tested for antipathogenic activity. Each *M. grisea* strain is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores for each strain are harvested into water with 0.01% Tween 20 to a concentration of $5x10^4$ spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100µg/ml. Solvent only is added to the second culture. Rice infection assays are performed using India rice cultivar CO39 essentially as described in Valent *et al.*, *supra*. Two-week-old seedlings of cultivar CO39 are sprayed with 12ml of conidial suspension. The inoculated plants are incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27°C 12 hours/21°C 12 hours 70% humidity) for an additional 5.5 days. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity of the mutant and wild-type fungal strains as compared to their untreated control samples.

Alternatively, antipathogenic activity can be assessed using an excised leaf pathogenicity assay. Spore suspensions are prepared in water only to a concentration of $5x10^4$ spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100μg/ml. Solvent only is added to the second culture. Detached leaf assays are performed by excising 1 cm segments of rice leaves from India rice cultivar CO39 and placing them on 1% agarose in water. 10μl of each spore suspension is place on the leaf segments and the samples are incubated at 25°C for 5 days in the dark. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity of the mutant and wild-type fungal strains as compared to their untreated control samples.

Example 12

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of Porphobilinogen Deaminase with Reduced Activity

The effect of test compounds on the growth of wild-type fungal cells and mutant fungal cells having a mutant PBG gene is measured and compared as follows. *Magnaporthe grisea* fungal cells containing a mutant form of the PBG gene resulting in reduced activity, such as a the transposon insertion mutation of cpgmra0012033e10 or a promoter truncation mutation that reduces expression, are grown under standard fungal growth conditions that are well known and described in the art. A promoter truncation is made by deleting a portion of the promoter upstream of the transcription start site using standard molecular biology techniques that are well known and described in the art (Sambrook *et al.*, *supra*).

The mutant and wild-type *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing heme, hemin, or protoporphyrin IX (Sigma) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores are added to each well of 96-well plates to which a test compound is added (at varying concentrations). The total volume in each well is 200μl. Wells with no test compound present (growth control), and wells without cells are

included as controls (negative control). The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild-type cells are screened under the same conditions.

The effect of each test compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound)/OD_{590} (growth control) x 100. The percent growth inhibition as a result of each of the test compounds on the mutant and wild-type cells is compared. Compounds that show differential growth inhibition between the mutant and the wild-type cells are identified as potential antifungal compounds. Similar protocols may be found in Kirsch & DiDomenico, *supra.*

Test compounds that produce a differential growth response between the mutant and wild-type fungal strains are further tested for antipathogenic activity. Each *M. grisea* strain is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores for each strain are harvested into water with 0.01% Tween 20 to a concentration of $5x10^4$ spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100μg/ml. Solvent only is added to the second culture. Rice infection assays are performed using India rice cultivar CO39 essentially as described in Valent *et al.*, *supra*. Two-week-old seedlings of cultivar CO39 are sprayed with 12ml of conidial suspension. The inoculated plants are incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27°C 12 hours/21°C 12 hours at 70% humidity) for an additional 5.5 days. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity of the mutant and wild-type fungal strains as compared to their untreated control samples.

Alternatively, antipathogenic activity can be assessed using an excised leaf pathogenicity assay. Spore suspensions are prepared in water only to a concentration of $5x10^4$ spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100μg/ml. Solvent only is added to the second culture. Detached leaf assays are performed by excising 1cm segments of rice leaves from India rice cultivar CO39 and placing them on 1% agarose in water. 10μl of each spore suspension is place on the leaf segments and the samples are incubated at 25°C for 5 days in the dark. Leaf samples are examined at 5 days post-inoculation to determine the

extent of pathogenicity of the mutant and wild-type fungal strains as compared to their untreated control samples.

Example 13

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of a Heme Biosynthetic Gene that Lacks Activity

The effect of test compounds on the growth of wild-type fungal cells and mutant fungal cells having a mutant form of a gene in the heme biosynthetic pathway is measured and compared as follows. *Magnaporthe grisea* fungal cells containing a mutant form of a gene that lacks activity in the heme biosynthetic pathway (*e.g.* porphobilinogen synthase or uroporphyrinogen-III synthase having a transposon insertion) are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing heme, hemin, or protoporphyrin IX (Sigma) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium containing heme, hemin, or protoporphyrin IX to a concentration of $2x10^5$ spores per ml.

Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild-type cells are screened under the same conditions.

The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of each of the test compounds on the mutant and the wild-type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild-type cells are identified as potential antifungal compounds. Similar protocols may be found in Kirsch & DiDomenico, *supra*.

Test compounds that produce a differential growth response between the mutant and wild-type fungal strains are further tested for antipathogenic activity. Each *M. grisea* strain is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores for each strain are harvested into water with 0.01% Tween 20 to a concentration of $5x10^4$ spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100μg/ml. Solvent only is added to the second culture. Rice infection assays are performed using India rice cultivar CO39 essentially as described in Valent *et al.*, *supra*. Two-week-old seedlings of cultivar CO39 are sprayed with 12ml of conidial suspension. The inoculated plants are incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27°C 12 hours/21°C 12 hours at 70% humidity) for an additional 5.5 days. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity of the mutant and wild-type fungal strains as compared to their untreated control samples.

Alternatively, antipathogenic activity can be assessed using an excised leaf pathogenicity assay. Spore suspensions are prepared in water only to a concentration of $5x10^4$ spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100μg/ml. Solvent only is added to the second culture. Detached leaf assays are performed by excising 1 cm segments of rice leaves from India rice cultivar CO39 and placing them on 1% agarose in water. 10μl of each spore suspension is place on the leaf segments and the samples are incubated at 25°C for 5 days in the dark. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity of the mutant and wild-type fungal strains as compared to their untreated control samples.

Example 14

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of a Heme Biosynthetic Gene with Reduced Activity

The effect of test compounds on the growth of wild-type fungal cells and mutant fungal cells having a mutant form of a gene in the heme biosynthetic pathway is measured and compared as follows. *Magnaporthe grisea* fungal cells containing a mutant form of a gene resulting in reduced protein activity in the heme biosynthetic

pathway (*e.g.* porphobilinogen synthase or uroporphyrinogen-III synthase having a promoter truncation that reduces expression), are grown under standard fungal growth conditions that are well known and described in the art. Mutant and wild-type *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing heme, hemin, or protoporphyrin IX (Sigma) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml.

Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild-type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of each of the test compounds on the mutant and wild-type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild-type cells are identified as potential antifungal compounds. Similar protocols may be found in Kirsch & DiDomenico, *supra*.

Test compounds that produce a differential growth response between the mutant and wild-type fungal strains are further tested for antipathogenic activity. Each *M. grisea* strain is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores for each strain are harvested into water with 0.01% Tween 20 to a concentration of $5x10^4$ spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100µg/ml. Solvent only is added to the second culture. Rice infection assays are performed using India rice cultivar CO39 essentially as described in Valent *et al.*, *supra*. Two-week-old seedlings of cultivar CO39 are sprayed with 12ml of conidial suspension. The inoculated plants are incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27°C

12 hours/21°C 12 hours at 70% humidity) for an additional 5.5 days. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity of the mutant and wild-type fungal strains as compared to their untreated control samples.

Alternatively, antipathogenic activity can be assessed using an excised leaf pathogenicity assay. Spore suspensions are prepared in water only to a concentration of $5x10^4$ spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100µg/ml. Solvent only is added to the second culture. Detached leaf assays are performed by excising 1 cm segments of rice leaves from India rice cultivar CO39 and placing them on 1% agarose in water. 10µl of each spore suspension is place on the leaf segments and the samples are incubated at 25°C for 5 days in the dark. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity of the mutant and wild-type fungal strains as compared to their untreated control samples.

Example 15

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Heterologous PBG Gene.

The effect of test compounds on the growth of wild-type fungal cells and fungal cells lacking a functional endogenous PBG gene and containing a heterologous PBG gene is measured and compared as follows. Wild-type *M. grisea* fungal cells and *M. grisea* fungal cells lacking an endogenous PBG gene and containing a heterologous PBG gene from *Saccharomyces cerevisiae* (Genbank Accession No. P28789), having 44% sequence identity, are grown under standard fungal growth conditions that are well known and described in the art.

A *M. grisea* strain carrying a heterologous PBG gene is made as follows. A *M. grisea* strain is made with a nonfunctional endogenous PBG gene, such as one containing a transposon insertion in the native gene that abolishes protein activity. A construct containing a heterologous PBG gene is made by cloning a heterologous PBG gene, such as from *Saccharomyces cerevisiae*, into a fungal expression vector containing a *trpC* promoter and terminator (*e.g.* Carroll *et al.*, 41 *Fungal Gen. News Lett.* 22 (1994) (describing pCB1003) using standard molecular biology techniques that are well known

and described in the art (Sambrook *et al.*, *supra*). The vector construct is used to transform the *M. grisea* strain lacking a functional endogenous PBG gene. Fungal transformants containing a functional PBG gene are selected on minimal agar medium lacking heme, hemin, or protoporphyrin IX, as only transformants carrying a functional PBG gene grow in the absence of heme, hemin, or protoporphyrin IX.

Wild-type strains of *M. grisea* and strains containing a heterologous form of PBG are grown under standard fungal growth conditions that are well known and described in the art. *M. grisea* spores are harvested from cultures grown on complete agar medium after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml.

Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The effect of each compound on the wild-type and heterologous fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of each of the test compounds on the wild-type and heterologous fungal strains are compared. Compounds that show differential growth inhibition between the wild-type and heterologous strains are identified as potential antifungal compounds with specificity to the native or heterologous PBG gene products. Similar protocols may be found in Kirsch & DiDomenico, *supra*.

Test compounds that produce a differential growth response between the strain containing a heterologous gene and strain containing a fungal gene are further tested for antipathogenic activity. Each *M. grisea* strain is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores for each strain are harvested into water with 0.01% Tween 20 to a concentration of $5x10^4$ spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100µg/ml. Solvent only is added to the second culture. Rice infection assays are

performed using India rice cultivar CO39 essentially as described in Valent *et al.*, *supra*. Two-week-old seedlings of cultivar CO39 are sprayed with 12ml of conidial suspension. The inoculated plants are incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27°C 12 hours/21°C 12 hours at 70% humidity) for an additional 5.5 days. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity of the wild-type and heterologous fungal strains as compared to their untreated control samples.

Alternatively, antipathogenic activity can be assessed using an excised leaf pathogenicity assay. Spore suspensions are prepared in water only to a concentration of $5x10^4$ spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100μg/ml. Solvent only is added to the second culture. Detached leaf assays are performed by excising 1cm segments of rice leaves from India rice cultivar CO39 and placing them on 1% agarose in water. 10μl of each spore suspension is place on the leaf segments and the samples are incubated at 25°C for 5 days in the dark. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity of the wild-type and heterologous fungal strains as compared to their control samples.

Example 16

Pathway Specific *In Vivo* Assay Screening Protocol

Compounds are tested as candidate antibiotics as follows. *Magnaporthe grisea* fungal cells are grown under standard fungal growth conditions that are well known and described in the art. Wild-type *M. grisea* spores are harvested from cultures grown on oatmeal agar media after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemocytometer and spore suspensions are prepared in a minimal growth medium and a minimal growth medium containing heme, hemin, or protoporphyrin IX (Sigma) to a concentration of $2x10^5$ spores per ml. The minimal growth media contains carbon, nitrogen, phosphate, and sulfate sources, and magnesium, calcium, and trace elements (for example, see innoculating fluid in Example 7). Spore suspensions are added to each well of a 96-well microtiter plate (approximately $4x10^4$ spores/well). For each well containing a spore

suspension in minimal media, an additional well is present containing a spore suspension in minimal medium containing heme, hemin, or protoporphyrin IX.

Test compounds are added to wells containing spores in minimal media and minimal media containing heme, hemin, or protoporphyrin IX. The total volume in each well is 200µl. Both minimal media and heme, hemin, or protoporphyrin IX containing media wells with no test compound are provided as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. A compound is identified as a candidate for an antibiotic acting against the heme, hemin, or protoporphyrin IX biosynthetic pathway when the observed growth in the well containing minimal media is less than the observed growth in the well containing heme, hemin, or protoporphyrin IX as a result of the addition of the test compound. Similar protocols may be found in Kirsch & DiDomenico, *supra*.

Published references and patent publications cited herein are incorporated by reference as if terms incorporating the same were provided upon each occurrence of the individual reference or patent document. While the foregoing describes certain embodiments of the invention, it will be understood by those skilled in the art that variations and modifications may be made that will fall within the scope of the invention. The foregoing examples are intended to exemplify various specific embodiments of the invention and do not limit its scope in any manner.